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14. ABSTRACT

The specific aim is to determine if breast cancer cells induce persistent gaps between aged endothelial cells, as compared to "young" endothelial cells, to cause increased cancer cell extravasation and metastasis. The objectives are: 1. To further elucidate the interactions of breast cancer cells with aged endothelial cells using co-cultures, 2. To investigate possible mechanisms of breast cancer cell-induced persistent gap formation in in-vitro aged endothelial monolayers, and 3. To begin examining the relevance of the in-vitro age-related differences in endothelial cell responses to breast cancer cell addition using rat in-vivo aging and metastasis models. In this final report, we find that: 1. Addition of rat breast cancer cells to microvascular endothelial cells harvested from young rats causes transient gaps between endothelial cells, whereas cancer addition to endothelial cells from old rats causes persistent gaps; 2. Early analysis shows that more breast cancer cells transmigrate endothelial cells harvested from young rats; and 3. Though metastases are larger in old rats, compared to young rats, after tail vein injection of cancer cells, differences in immune function may be a confounding factor as cancer cell injection the mammary fat pads causes larger "tumors" in the old rats.

15. SUBJECT TERMS
endothelial cells, cell culture, rat models, extravasation, apoptosis, aging, reactive oxygen species, cell biology

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Appendix B: Merkle, C.J., Kuster, P.A., Vidrine, A.N., Brown, M.T., Little, T.H., Alderette, A., and Montgomery, D.W., Responses of in-vitro aged endothelial cells to breast cancer cell addition: Role of endothelial-produced reactive oxygen species in intercellular gap formation and cell death, submitted manuscript

Introduction

The risks for developing and dying from breast cancer increase with age. Death from breast cancer is usually due to metastatic disease. Key events in the metastatic cascade process involve breast cancer cell passage through endothelial cells. In cell culture experiments, we have observed that breast cancer cells cause younger (lower passage) endothelial cells to form transient gaps between neighboring cells, while the addition of breast cancer cells to older (higher) passage endothelial cells causes persistent gaps between neighboring endothelial cells. Since these differences may have relevance to metastasis and age-related increases in BR CA mortality, we have continued to investigate age-related responses of endothelial cells to BR CA addition. The objectives of this research project are:

1. To further elucidate the interactions of breast cancer cells with aged endothelial cells using co-cultures
2. To investigate possible mechanisms of breast cancer cell-induced persistent gap formation in in-vitro aged endothelial monolayers
3. To begin examining the relevance of the in-vitro age-related differences in endothelial cell responses to breast cancer cell addition using rat in-vivo aging and metastasis models

Body

Task 1. To determine whether more MCF-7 breast cancer cells will transmigrate through monolayers of old endothelial cells than through monolayers of young endothelial cells. (Months 1-6)

This task was completed and originally addressed in the May 2003 annual report. We found that more MCF-7 breast cancer cells transmigrate old endothelial cells, compared to young endothelial cells, plated on porous filters in transwell chambers. The findings of this task have been published (Merkle, C.J., Torres, B.J., Baruch J.M., Stevens, K., Munoz, C., Schaeffer, R.C., & Montgomery, D.W. 2005. In- vitro age related responses of endothelial cells to cancer cell addition. Cancer Detection and Prevention, 29, 518-527).

Task 2. To determine if persistent breast cancer-induced gaps between old endothelial cells are due to alterations in quantity and/or intracellular distribution of N-, VE- and pan-cadherins. (Months 7-12)

This task was completed and was addressed in the May 2004 annual report. In brief, we found that old endothelial cells have less N- and VE-cadherin in no added cancer cell controls and experimental samples in which MCF-7 breast cancer cells are added. These results have been presented in a poster format at national meetings (ERA of Hope and Annual Meeting of American Society for Cell Biology). We plan to combine the findings with findings regarding differences in endothelial cell migration, discussed below, and publish them.

Task 3. To determine if BR CA cell-induced gaps in old endothelial cells are due to apoptosis. (Months 7-12)

This task was completed and was addressed in the May 2003 report. In summary, two assays (annexin V binding and an electrophoresis-based DNA fragmentation assay) supported that the MCF-7 breast cancer cells induced apoptosis in the old, but not the young, endothelial cells. The results of a caspase-3 assay were inconsistent. These results were published (Merkle, C.J., Torres, B.J., Baruch J.M., Stevens, K., Munoz, C., Schaeffer, R.C., & Montgomery, D.W. 2005. In- vitro age related responses of endothelial cells to cancer cell addition. Cancer Detection and Prevention, 29, 518-527).

Task 4. If old endothelial cells do not demonstrate an increased rate of apoptosis compared to young endothelial cells in response to breast cancer cell addition (Task 3), we will determine if gaps persist longer in old cells than young cells due to their lower rate of cell proliferation. (Months 13-18)

This task was completed and was addressed originally in the May 2004 annual report. In summary, we found, as have others, that endothelial cells aged in culture had a reduced proliferation rate. However, in our co-culture experiments the reduction in proliferation rate did NOT account for the persistent gaps between old endothelial cells after breast cancer cell addition. These findings appear in detail in a submitted manuscript that is attached to this report (Merkle, C.J., Kuster, P.A., Vidrine, A.N., Brown, M.T., Little, T.H., Alderette, A., and Montgomery, D.W., Responses of in-vitro aged endothelial cells to breast cancer cell addition: Role of endothelial-produced reactive oxygen species in intercellular gap formation and cell death, submitted manuscript).

Task 5. If old endothelial cells do demonstrate an increased rate of apoptosis in response to breast cancer cell addition (task 3), we will determine the roles of H_2O_2 and Fas/Fas ligand in apoptosis induction (months 13-30)

This task was completed and addressed in both the May 2004 and May 2005 annual reports. In summary, we have found that old endothelial cells are more sensitive to ROS and that antioxidants partially block MCF-7 cell-induced endothelial cell gap formation. A surprise finding was that the increase in ROS is due to increased production by the endothelial cells, and not the MCF-7 breast cancer cells. These findings appear in a manuscript that has been submitted to a journal for review (Merkle, C.J., Kuster, P.A., Vidrine, A.N., Brown, M.T., Little, T.H., Alderette, A., and Montgomery, D.W., Responses of in-vitro aged endothelial cells to breast cancer cell addition: Role of endothelial-produced reactive oxygen species in intercellular gap formation and cell death, submitted manuscript).

Task 6. If Fas/Fas ligand and H_2O_2 are not implicated in endothelial cell apoptosis, we will determine if apoptosis is due to a failure of cells to spread and reattach after gap formation (months 24-30)

A number of experiments were performed in which young and old BPAECs grown in culture flasks were imaged over time (for 2 hour intervals beginning at various time points) after the addition of CFDASE-labeled MCF-7 BR CA cells. In these studies, the cancer cells were labeled with CFDASE, then added to the flasks of BPAECs. Then the flasks were kept at 37 degrees centigrade by the heated microscope stage. Initial images were obtained using the fluorescence optics and combined fluorescence-phase contrast illumination to identify the cancer cells and distinguish them from the BPAECs. The specific area of the flask was then observed for two hours with images captured every 2.5 minutes. After the observation period, images were "stacked" and software was used to create "movies". All of these experiments were completed some time ago.

Over the past year and one-half, we have been quantifying a number of variables from these "movies". These variables include: 1. gap size, 2. cell movement (total and directional), 3. cell retraction and spreading, 4. number of cells undergoing mitosis, and 5. number of cells undergoing apoptosis. Qualitative observations at some time points have suggested that the young BPAECs overall are very active and motile compared to the old BPAECs. Extraction of these data from the "movies" has proven to be very time consuming, but we have determined a number of significant findings. These data suggest that the younger BPAECs are moving farther from their origin, though rate of movement and total distance traveled are the same. We continue to "mine" information and plan to combine these data with data from Task 2 to write a final paper from this grant.

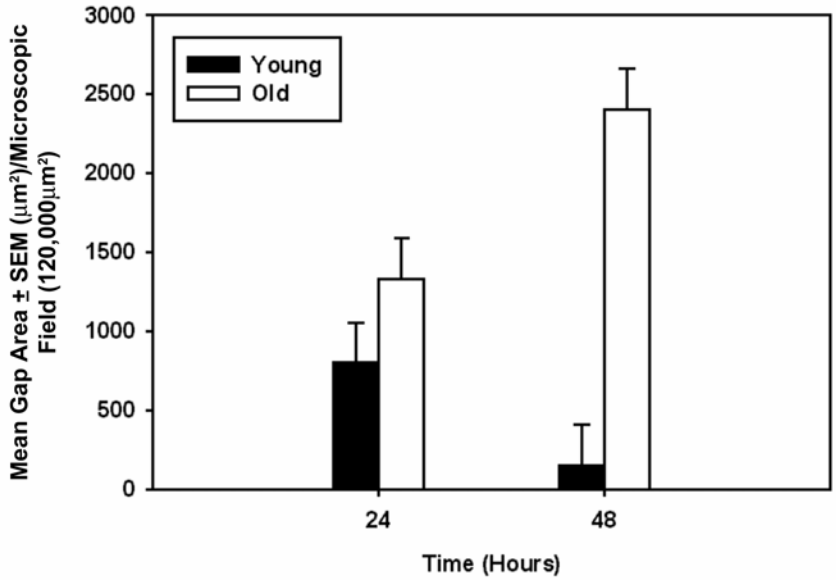
Problems with examining focal adhesion kinase (FAK) in cell spreading and motility in young and old endothelial cells were identified in the May 2005 annual report. Due to the inability of my staff to capture images in focus before loss of the fluorescence signal, this project was not completed.

Task 7. Determine if more breast cancer transmigrate endothelial cell monolayers of cells harvested from old rats compared to cells harvested from young rats (months 19-24)

We perfected isolation of microvascular endothelial cells from the lungs of young and old rats. The following experiment is in progress and will be completed next week. The endothelial cells isolated from the young and old rats are plated on on FluoroBlok inserts of BD BioCoat™ Tumor Invasion Systems. CFDASE-labeled MADB106 breast cancer cells are added at 20,000 cells per well, then incubated for 24 hours. In some wells, the MADB106 cells are added without endothelial cells plated on the filters. Relative fluorescence associated with the numbers of MADB106 cells that cross the porous filter and enter the bottom chamber are measured using a Tecan plate reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Thus far, there is a statistically significant difference between the fluorescence associated with MADB106 breast cancer cells crossing filters covered with microvascular endothelial cells from old rats compared to young rats. More fluorescence is associated with the samples of endothelial cells from old rats compared to young rats (mean = 41,347 $\pm$ 3,763 in samples containing endothelial cells from young rats versus mean = 46,269 $\pm$ 3,079 for endothelial cells from old rats, t test shows $p < 0.01$).

Since it was not possible to clearly view the rat endothelial cells on the porous filters, we plated endothelial cells harvested from young and old rats on chamber slides to better observe gaps induced by the MADB106 breast cancer cells. After the endothelial cells achieved confluent, fluorescently-labeled MADB106 cells were added. The co-cultured samples were fixed 24 and 48 hours later, stained with fluorescent phalloidin (to observe gaps between endothelial cells more easily) and observed by fluorescence microscopy. Gap area between the endothelial cells was measured from digital images using a computer program. As shown in the graph below, gap area between endothelial cells harvested from old rats (empty bars) was very large 48 hours after MADB106 breast cancer cell addition, while gaps were small 48 hours after the addition of the breast cancer cells to endothelial cells harvested from young rats (solid bars). These results are consistent with findings in cultured bovine endothelial cells and MCF-7 breast cancer cells that showed transient gaps between young endothelial cells and persistent gaps between aged endothelial cells.

Task 7 Results

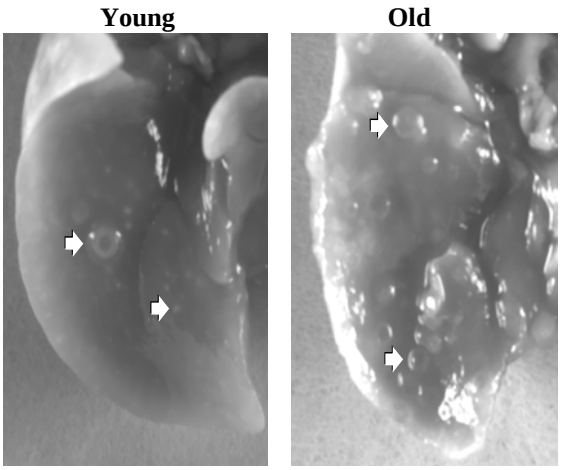


Task 8. Determine if more lung metastases occur in old rats than in young rats after IV injection of MADB106 BR CA cells (Months 25-36)

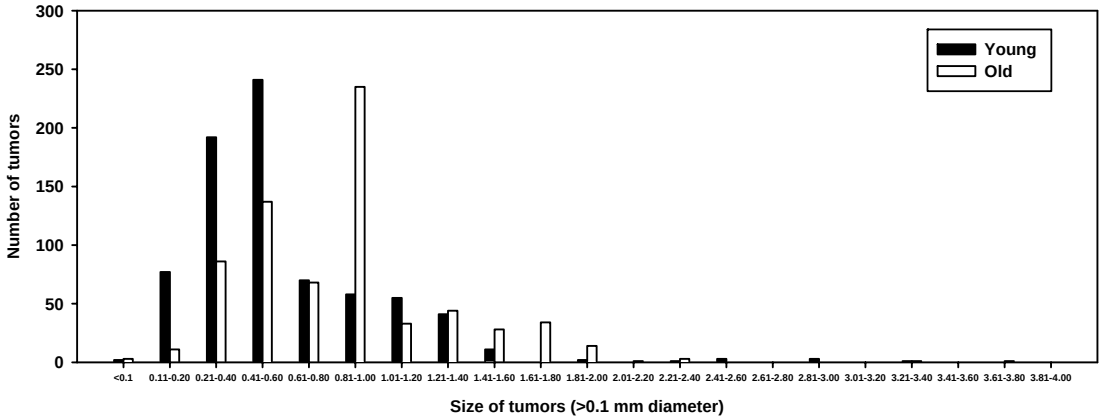
As reported earlier, these experiments were begun in September 2004 and Task 8 was completed in May 2005. We determined that there was no statistical difference in the total number of lung metastases in young (3 month old) and old (24 month old) rats 2 weeks after the syngenic MADB106 BR CA cells were injected into the tail vein. (See Figure A on following page for views of the lungs.) However, the mean size of the lung metastases per rat was larger (0.56 $\pm$ 0.25 millimeters for young rats, compared to 0.81 $\pm$ 0.43 for old rats [p = 0.014]), and the overall size of the lung metastasis was larger in old rats (0.64 $\pm$ 0.43 for young rats, compared to 0.89 $\pm$ 0.45 for old rats [$p < 0.0001$]). Furthermore, examination of the distribution of lung metastases based on size suggested that the young rats had larger numbers of smaller tumors, while the old rats had more of the larger tumors (see Figure B on the following page). Further analysis (see Figure C) showed statistical differences in the number of metastases less than or equal to 0.7 mm, with the young rats having more of these smaller tumors. Older rats had more metastases greater than 0.7 mm.

Task 8 Results

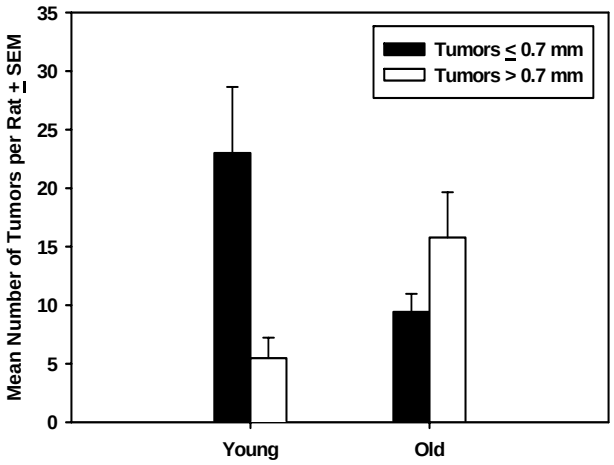
A



B



C



It is possible that differences in immune function between the young and old rats accounts for the differences in metastatic tumor size. Thus, we injected MADB106 breast cancer cells into the mammary fat pads of young and old rats, then measured "tumor" size 10 days later. Since the mammary fat pad tumors were larger in the old rats, it is possible that differences in immune function account for the differences in metastatic tumor size. We are hoping that Task 9 below will help resolve this issue.

Task 9. Determine the extent of MADB106 extravasation in the lungs of 3-month old and 24-month old rats (Months 31-36)

This task was proposed to be done if there were differences in metastatic tumor number. As stated above, we found differences in size not number. However coupled with the finding that tumor "size" of the MADB106 cells injected into the mammary fat pad were larger in the older rats, we decided that the transmigration experiments would be helpful in determining if we could identify increased numbers of breast cancer cells exiting the vasculature in histology slides. Furthermore, the appearance of NK cells attacking the MADB106 cells in the vasculature of the young, but not the old rats, would be helpful in supporting or not supporting immune cell differences. Hence, this week we are injecting young and old rats solely for the purpose of harvesting lung tissue for histological examination.

Key Research Accomplishments

We perfected a procedure for isolating endothelial cells from the lungs of young and old rats. These cells were used to conduct experiments on chamber slides and transwell chambers. The results of co-culture experiments employing MADB106 cells and microvascular endothelial cells from young and rats were similar to those obtained using the in-vitro aged bovine endothelial cell-MCF-7 breast cancer cell model. Additionally, the early results from the transwell filter studies show that more MADB106 breast cancer cells cross endothelial cells from old rats compared to young rats. Importantly, our results demonstrate that our original findings made using a cell culture bovine endothelial aging model and human MCF-7 breast cancer cells appears to have relevance in vivo.

Final Reportable Outcomes

1. Manuscripts

A. Merkle, C.J., Torres, B.J., Baruch J.M., Stevens, K., Munoz, C., Schaeffer, R.C., & Montgomery, D.W. 2005. In- vitro age related responses of endothelial cells to cancer cell addition. Cancer Detection and Prevention, 29, 518-527.

B. Merkle, C.J., Kuster, P.A., Vidrine, A.N., Brown, M.T., Little, T.H., Alderette, A., & Montgomery, D.W., Responses of in-vitro aged endothelial cells to breast cancer cell addition: Role of endothelial-produced reactive oxygen species in intercellular gap formation and cell death, submitted manuscript.

C. Merkle, C.J., Vidrine, A.N., & Montgomery, D.W. Breast Cancer Invasion and Metastasis in Young and Old Rats, in preparation.

2. Abstracts

A. Merkle, C.J., Vidrine, A.N. & Montgomery, D.W.. 2005. Age-dependent loss of endothelial motility and breast cancer-induced endothelial gaps. Biological Research for Nursing 6: 310-311.

B. Merkle, C.J., Vidrine, A.N., Johnson, R.J., Kuster, P.A., & Montgomery, D.W.. 2004. Reactive oxygen intermediates and age-related differences of endothelial cell responses to breast cancer cells. 441a. This citation/ abstract was verified on 5-31-05 at the following web address: http://www.ascb.org/meetings/am2004/pdfs/abbook04.pdf.

C. Merkle, C.J., Stevens, K., Munoz, C., Hussain, S., Schaeffer, R.C., & Montgomery, D.W.. 2002. In-vitro age-dependent responses of endothelial cells to breast cancer cell addition. Molecular Biology of the Cell 13: 549a.

D. Merkle, C.J., Stevens, K.R., & Montgomery, D.W. 2003. Aged endothelial cells facilitate breast cancer transmigration in-vitro. Communicating Nursing Research Conference Proceedings 36: (WIN Assembly 11:) 159.

E. Merkle, C.J., Kuster, P.A., Little, T.H., Vidrine,A.M., Brown, M.T., & Montgomery, D.W. 2003. Changes in VE-cadherin may have a role in age-dependent differences in endothelial responses to breast cancer cell addition. Molecular Biology of the Cell 14: 549a.

F. Merkle, C.J., Brown, M.T., Vidrine, A.N., & Montgomery, D.W. 2003. Cell division is not required to close breast cancer-induced endothelial gaps. Communicating Nursing Research Conference Proceedings 37: (WIN Assembly 12:) 170.

3. Invited Talks

Merkle, C.J. 2005. Vascular aging and breast cancer metastasis, Mary Martha and Ed Stinnett Visiting Scholar Lectureship; University of Texas School of Nursing, Houston, TX

Conclusions

Our conclusions from this year's work are that:

1. Based on the results of experiments performed on chamber slides and early analyzed experiments performed in transwell chambers, it appears that endothelial cells isolated from the lungs of old rats are induced to form persistent gaps and to permit the passage of more MADB106 breast cancer cells past them, in comparison to endothelial cells isolated from the lungs of young rats. These findings support the applicability of our initial observations made by aging endothelial cells in vitro. In view of the more recent work of the Cheresh group, a

mechanism that may account for these differences relates to VEGF. VEGF produced by cancer cells is known to cause VE-cadherin to “unzip”. A very logical set experiments to perform would involve the responses of aged endothelial cells to VEGF and to see if gap formation can be blocked by antibodies against VEGF.

2. Using the tail vein injection model, the numbers of lung metastases were the same in young and old rats. However, the old rats had larger metastases. Furthermore, the young rats had more small tumors (less than or equal to 0.7 mm), whereas old rats had more larger metastases (greater than 0.7 mm). This is consistent with the idea that an aged endothelium permits the transmigration of more breast cancer cells. However, since injection of the MADB106 breast cancer cells into the mammary fat pads of old rats causes larger tumors than injection into young rats, the immune system differences in old rats could contribute to the larger metastastases. Thus, additional work needs to be done to separate the influences of an aging immune system from an aging vasculature. These experiments should focus on the NK cells, as this cell type is the primary responded to injected MADB106 breast cancer cells in the tail vein injection rat model.

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In vitro age-related responses of endothelial cells to breast cancer cell addition

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Abstract

Aim: The purpose of this study was to determine if the in vitro age of endothelial cells alters endothelial response(s) to breast cancer cells. *Method:* After characterizing lower passage (''young''; passages 10–16) and higher passage (''old''; passages 30–36) bovine pulmonary artery endothelial cells (BPAECs), fluorescently labeled MCF-7 breast cancer cells were added to confluent monolayers of young and old BPAECs. *Results:* Transient gaps that peaked in size by 12 h and closed by 48 h occurred between the young BPAECs, while large persistent gaps formed between the old BPAECs. Gap formation did not occur when 184A1 cells, a non-malignant mammary epithelial cell line, were added in place of MCF-7 cells, suggesting that the age-related responses of the endothelial cells to MCF-7 cell addition were specific to the tumor cell addition. Additionally, more MCF-7 cells migrated through old BPAEC monolayers, than young BPAEC monolayers, grown on Matrigel™-coated filters. Finally, DNA fragmentation and fluorescent annexin-V binding assays suggested increased MCF-7 cell-induced apoptosis in older BPAECs, though results from a caspase-3 activation assay were equivocal. *Conclusions:* In sum, our findings support the notion that aged endothelial cells are more susceptible to breast cancer-induced injury, perhaps due to increased apoptosis.

Keywords: Apoptosis; Metastasis; Microscopy; Gap formation; Endothelial aging model; Co-culture experiments; Bovine pulmonary artery endothelial cells (BPAEC); MCF-7 cells; Breast cancer cells; In vitro response

1. Introduction

In the year 2005, it is predicted that 40,870 Americans will die from breast cancer [1]. Death from breast cancer, as well as many other solid tumors, usually results from metastatic disease. Metastasis involves interactions between cancer cells and endothelial cells of the blood and lymph vasculature [2]. Interactions that occur as breast cancer cells exit the vasculature when colonizing distant organs are not completely understood, though at some point the cancer cells pass through gaps between previously joined endothelial cells. The importance of gaps in metastasis is suggested by a recent in vitro study demonstrating that vascular endothelial growth factor (VEGF) treatment caused inter-endothelial gap formation and promoted breast cancer cell transmigration [3].

Co-culture studies employing melanoma cells and human umbilical vein endothelial cells (HUVECs) have enabled identification of events in tumor transendothelial migration that involve tumor penetration of endothelial cell–cell contacts followed by N-cadherin-dependent endothelial resealing over the transmigrated tumor cell [4,5]. Other in vitro work suggests that breast cancer cells and osteosarcoma cells induce apoptosis in endothelial cells [6,7], which could potentially leave gaps in the endothelial layer. Both observations may be applicable to the

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mechanisms by which breast cancer cells create gaps in the endothelium during metastasis.

Aging may also play a role in cancer metastasis. It was recently reported that the incidence of lymph node metastasis was significantly greater in colorectal cancer patients more than 80 years of age than in those between 40 and <80 years old. Those over 80 years of age also had a poorer survival rate [8]. Using a mouse model of transplantable breast cancer, Gravekamp et al. [9] found that the incidence of cancer metastasis was twice as great in old (21 months) mice as in young (3 months) mice. Therefore, in view of the important role of vascular endothelium in cancer metastasis, these observations suggest that endothelial aging might also play a role in this process.

The purpose of the present study was to determine if the in vitro age of endothelial cells alters endothelial response(s) to breast cancer cells. An in vitro endothelial aging model was developed and used in co-culture experiments to study in vitro age-related endothelial responses to breast cancer cell addition. Indeed, we found that MCF-7 breast cancer cell-induced gap formation between endothelial cells is sensitive to the in vitro age of the endothelial cells. Transient gaps between endothelial cells occurred when breast cancer cells were added to ''younger'' (passage 10–16) endothelial cells. Large persistent gap formation between in vitro aged endothelial cells (passage 30–36) occurred after breast cancer cell addition. Additionally, in co-culture experiments performed in bioinvasion chambers, fewer MCF-7 breast cancer cells crossed lower passage endothelial cells grown on the Matrigel™-coated porous filters of the invasion chambers than the higher passage endothelial cells. Finally, findings from DNA fragmentation and annexin-V binding assays suggested that the persistent larger gaps between older endothelial cells may be due in part to apoptosis, perhaps through a caspase-3 independent pathway, based on equivocal findings from caspase-3 activation assays.

2. Materials and methods

2.1. Cells

Three cell lines were used in these studies. These were: (1) bovine pulmonary artery endothelial cells (BPAECs) from a personal stock, which had been previously characterized [10], (2) MCF-7 breast cancer cells (American Type Culture Collection, Manassas, VA), and (3) 184A1 cells (American Type Culture Collection, Manassas, VA), a non-malignant human mammary duct epithelial cell line. Cells were grown in 75 cm^2 culture flasks at 37 °C in 7.5% CO_2. BPAECs and MCF-7 cells were grown in Dulbecco's Modified Eagle's Media (DMEM) containing 10% fetal calf serum (FCS; HyClone Laboratories, Logan, UT), penicillin (100 units/ml), streptomycin (0.1 mg/ml) and gentamicin (50 μg/ml). The 184A1 cells were grown in Mammary Epithelial Cell Growth Medium (Clontech) containing pituitary extract. Cells were passed using Hanks EDTA solution and trypsin. When passing the BPAECs, half of the collected cells were replaced into the flask for continued propagation.

2.2. Endothelial aging model

After sequentially subculturing BPAECs, two in vitro ''age'' groups were characterized. The two groups consisted of (1) lower passage ''young'' cells, which included passages 10–16, and (2) higher passage ''old'' in vitro aged cells, passages 30–36. Light microscopy was used to characterize the groups by morphological features. Images of confluent monolayers of young and old BPAECs were digitally captured using a SPOT camera (Diagnostic Instruments, Inc.) attached to a microscope (Leica DMIRB) using a 10× objective and phase-contrast optics. Image analysis software (Image-Pro™ Plus, Media Cybernetics) was used to collect the following data: (1) cell density per microscopic field; (2) cell area, determined by tracing the cell perimeter; (3) number of oversized cells, defined as cells having an area greater than 3500 μm^2; (4) area of oversized cells.

In addition to morphological characterization, a telomere restriction length assay was performed on low and high passage BPAECs using a commercially available kit (Telomere Fragment Length Assay Kit, Pharmingen) to determine if telomere fragment length was shorter in the old BPAECs in comparison to the young BPAECs. In this assay, a Southern blot was performed using a telomere sequence-specific probe following kit instructions. In addition to examining telomere fragment length of young and old BPAECS using the TeloHi and TeloLo standards of the kit, telomeres from Jurkat cells, a human T-cell lymphoma cell line (American Type Culture Collection), were examined as a control.

2.3. Co-culture experiments

In studies in which data were to be obtained using microscopy, young and old BPAECs were grown to confluence on fibronectin-coated gelatinized plastic 8-well chamber slides. After the BPAECs attained a ''cobblestone'' appearance, MCF-7 cells, which were labeled with carboxyfluorescein diacetate, succinimidyl ester (CFDASE; Vybrant™ CFDA SE Cell Tracer Kit, Molecular Probes, Eugene, OR), were added (2500 cells per well) to experimental cultures and fresh culture medium was added to controls. Samples were fixed with 5% formaldehyde at 4, 6, 12, 24 and 48 h after MCF-7 cell addition and mounted for microscopy. In an experiment designed to control for the breast cancer cell addition, CFDASE-labeled 184A1 cells (2500 cells per well) were added to some wells and samples were fixed 24 h after cell addition.

In assays that required larger numbers of cells, BPAECs were grown on gelatinized 75 cm^2 culture flasks. For

co-culture experiments performed in flasks, MCF-7 and 184A1 cells were added at 200,000 cells per flask of confluent endothelial cells. In experiments performed on Matrigel™-coated filters (with 8 μm pores) in transwell invasion chambers (24-well plate size; Becton Dickinson Labware, Bedford, MA), BPAECs were plated at 80,000 cells per well, while CFDASE-labeled MCF-7 cells were added at 100,000 cells per well after the BPAECs achieved confluence. Experiments were performed multiple times.

2.4. Microscopy and image analysis of co-culture experiments

In the experiments performed on chamber slides, 16 images (4 per well, 4 wells per experimental condition) were randomly obtained using combined phase-contrast and fluorescence microscopy (using a dual rhodamine-fluorescein filter and 40× objective) by personnel unfamiliar with the experimental conditions. Image analysis was performed using Image-Pro™ Plus software. In the initial co-culture experiments, gap size was determined by tracing the gaps between BPAECs with the computer mouse, then using the software to calculate area. In later experiments, prior to mounting chamber slides, formaldehyde-fixed samples were permeabilized with triton-X 100, then stained with rhodamine-phalloidin (Molecular Probes). Companion color and black-and-white images were obtained. Numbers of MCF-7 cells and 184A1 cells, which fluoresced green due to CFDASE labeling, were counted in the color images. The black-and-white images were used to measure gap area. Gap area was determined using a macro in which a ''flood'' mode was used to calculate gap area. Gaps appeared black and could be differentiated from non-gap area by differences in fluorescence intensity due to rhodamine-stained actin in cells. In these area measurements, pixels were converted to microns by calibration with an image of a micron graticule.

2.5. Bioinvasion chamber assays

Young and old BPAECs were plated onto the Matrigel™-coated filters of invasion chambers, and the BPAECs were grown until they attained confluence (between 24 and 48 h). CFDASE-labeled MCF-7 cells (80,000 cells per top chamber) were then added in the designated co-cultured samples, and labeled MCF-7 cells were added to control chambers without BPAECs. Twenty-four hours later the filters were fixed and the top surface scrubbed according to the manufacturer's instructions. Using fluorescence microscopy, the numbers of MCF-7 cells that entered the bottom chambers were determined by counting the number of cells on the underside of the filter and the plate well bottom. Data obtained from multiple experiments were pooled so that there were data from 11 invasion chambers for each of the three conditions, i.e. MCF-7 cells added to filters without BPAECs, MCF-7 cells added to filters covered by young BPAECs and MCF-7 cells added to filters covered by old BPAECs.

2.6. Apoptosis assays

Three types of assays were used to measure apoptosis. First, a DNA fragmentation assay was performed to detect DNA ladders, as previously described [11]. In these experiments, MCF-7 cells were added to young and old BPAECs grown in 75 cm^2 culture flasks. In this assay, MCF-7 cells plated alone were used as a negative control and BPAECs treated with 1 mM hydrogen peroxide for 4 h were used as a positive control. Second, fluorescein annexin-V binding (using ApoAlert™ Annexin-V-FITC, Clontech Laboratories) and propidium iodide were used to identify apoptotic cells in experiments performed on chamber slides, as previously described [11]. In these studies, CFDASE-labeled MCF-7 cells were added to young and old BPAECs 24 h prior to performing the annexin-V binding assay. Young and old BPAECs were examined without MCF-7 cell addition as negative controls. Lastly, caspase-3 activation was measured using a commercially available assay following kit instructions (EnzChek Caspase-3 Assay Kit #1, Molecular Probes) in co-culture studies performed in culture flasks.

2.7. Statistical analyses

Data were entered on spread sheets and analyzed using SigmaStat™ (Jandel). Means were determined and compared using the Student's *t*-test, one-way ANOVA or two-way ANOVA, as appropriate, if the data were normally distributed. If data violated normality, the Mann–Whitney Rank Sum Test, Kruskall–Wallis Rank Sum Test and Kruskall–Wallis ANOVA on Ranks were performed. As appropriate, the Student–Neuman–Keuls test was used for post hoc analyses. Statistical significance was set at $p < 0.05$.

3. Results

3.1. In vitro endothelial aging model

For these studies, the first task was to develop and characterize an endothelial aging model using BPAECs. As shown in Fig. 1A, young BPAECs, which were those grown to passages 10–16 (passage 15 shown), were small single-nucleated cells that attained a ''cobblestone'' appearance after forming a confluent monolayer. Occasionally, ''oversized'' cells, defined as cells having an area greater than 3500 μm^2 after tracing the perimeter, were seen. When BPAECs were sequentially subcultured to passages 30–36, forming the old endothelial cell population, many changes occurred, as apparent in Fig. 1B (passage 34 shown). First, cell area increased so that many ''oversized'' cells were identified per microscopic field, and consequently the

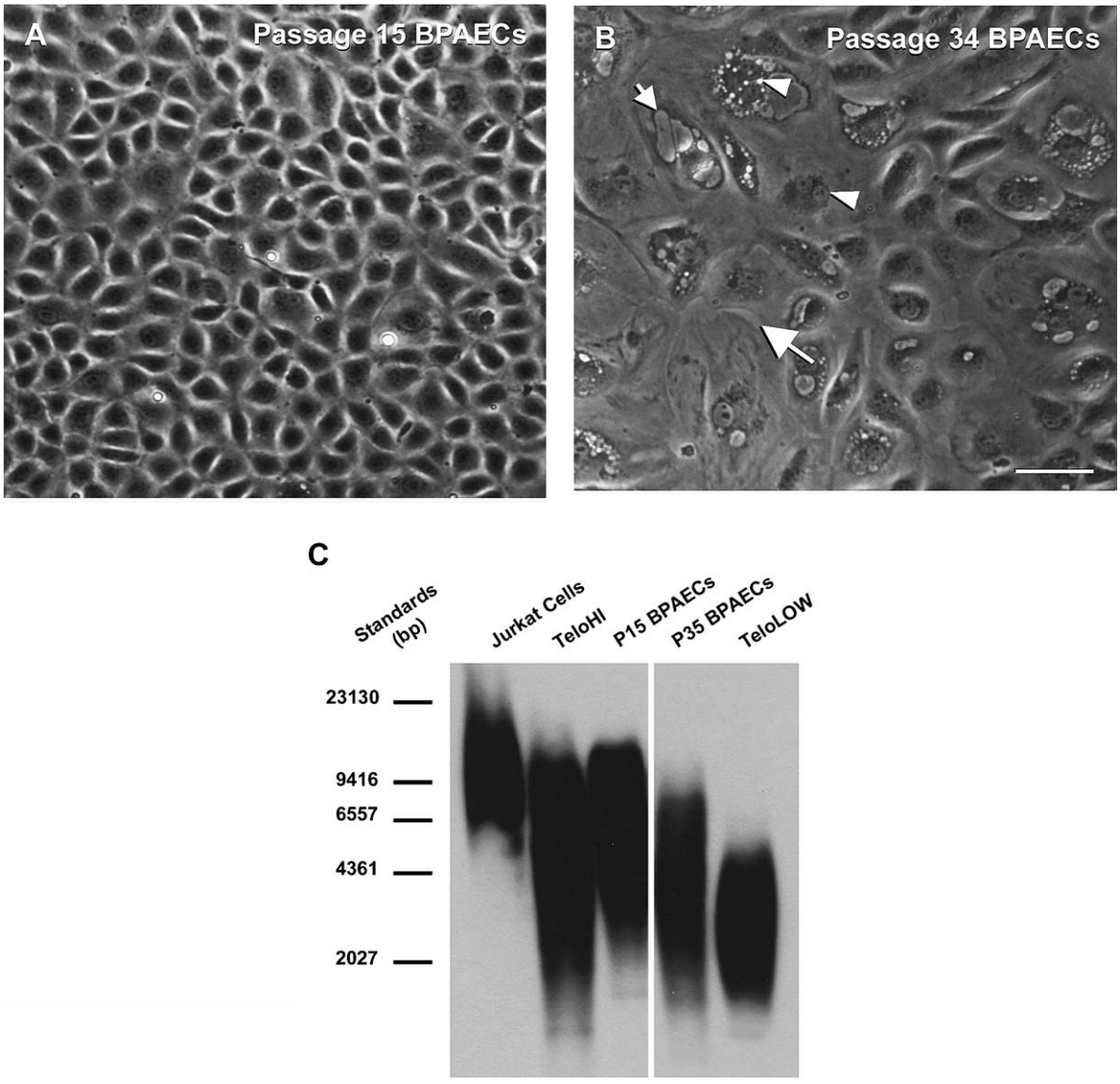


Fig. 1. Differences between young, passages 10–16, and old, passages 30–36, BPAECs. (A) Phase-contrast micrograph of live young, passage 15 BPAECs. (B) Micrograph of live old, passage 34, BPAECs. In old BPAECs, multi-nucleated cells (arrowhead), vesicles (small arrows), and small gaps between cells (large arrow) are more common. Bar equals 50 μm. (C) Telomere restriction length (TRL) assay. In the gel, high (TeloHi) and low (TeloLOW) molecular weight mass TRL sequence standards are shown in comparison with TRLs from early and late passage BPAECs.

number of cells per field (cell density) decreased. Secondly, a number of the old BPAECs had multiple nuclei (arrowheads in Fig. 1B) and large vesicles (small arrow).

A summary of the morphological characteristics of young and old BPAECs obtained from analysis of microscopic images appears in Table 1. Differences in numbers of cells per field, cell area, number of oversized cells, and area of the oversized cells reached statistical significance ($p < 0.05$). Except for cell number per microscopic field, non-parametric tests were used since the data derived from the old BPAECS were not normally distributed. This is illustrated by the discrepancies between mean and median values for the old BPAECs. Furthermore, old BPAECs had reduced telomere fragment length. In the assay shown in Fig. 1C, the mean terminal restriction fragment length decreased from 6520 to 3893 base pairs between passages 15 and 35 BPAECs. Thus, the selected passage numbers used to define young and old BPAECs formed groups of cells with morphologically distinct features.

3.2. *In vitro age-related responses of BPAECs to MCF-7 cell addition*

In these studies, young and old BPAECs were grown to confluence. CFDASE-labeled MCF-7 cells were added (2500 cells per well) to treatment wells. As controls, some wells had no added MCF-7 cells, while in other wells conditioned culture medium from growing MCF-7 cells was added. As shown in Fig. 2A and D, by 4 h after MCF-7 cell addition to young (passage 15 BPAECs shown) and old

Table 1
Morphometric characteristics of young and old BPAECs

	Young BPAECs				Old BPAECs				
Characteristic	*N*	Mean	S.D.	Median	*N*	Mean	S.D.	Median	*P*
Number of cells/field	19[a]	891	152	886	13[a]	253	144	197	<0.01[c]
Cell area (μm^2)	475[b]	952	436	854	325[b]	3030	4186	1791	<0.05[d]
Number of oversized cells/field	19[a]	3.5	3.2	3.0	13[a]	16.3	13.7	7.0	<0.05[d]
Oversized cell area (μm^2)	67[b]	5617	2523	5034	212[b]	11140	8713	8283	<0.05[d]

[a] *N* refers to the number of 1 mm^2 microscopic fields counted.
[b] *N* refers to the number of randomly selected cells measured.
[c] Data were analyzed using the Student's *t*-test.
[d] Data were analyzed using the Mann-Whitney Rank Sum Test.

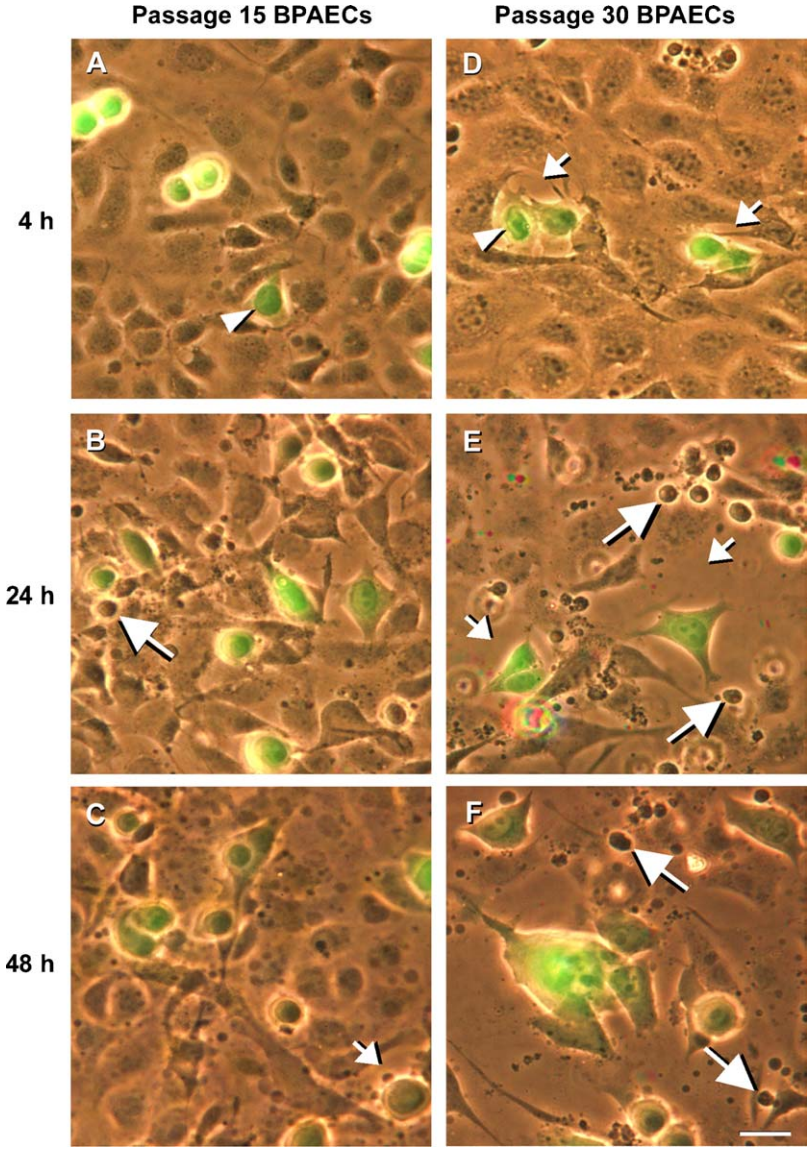


Fig. 2. Differences in MCF-7 cell-induced gap formation between young and old BPAECs. Images obtained using combined phase-contrast and fluorescence microscopy showed that MCF-7 cells (green; arrowheads) induced gaps (small arrows) between BPAECs by 4 h after MCF-7 cell addition. Gaps between young and old BPAECs were large 24 h later, but gaps closed by 48 h in the samples containing young BPAECs (compare (C)–(F)). Larger arrows mark rounded fragments that resembled apoptotic bodies; bar equals 20 μm.

BPAECs (passage 30 BPAECs shown), the MCF-7 cells adhered and spread (arrowheads). Small gaps were detected between the endothelial cells at the site of cancer cell adhesion (small arrows). At 24 h after MCF-7 cell addition, larger gaps were seen between both the young and old BPAECs (Fig. 2B and E). Also rounded cell fragments, which resembled apoptotic bodies, were observed (large arrows). By 48 h after addition however, gaps between neighboring young BPAECs were small, while gaps between old BPAECs were very large (compare Fig. 2C–F).

Since gap area between older BPAECs in control cultures was larger than that between younger BPAECs, the data were normalized by subtracting the mean area determined in controls having no cancer cells added. A graph showing the time course for mean gap area after MCF-7 cell addition to young and old BPAECs is presented in Fig. 3, and analysis of these data by two-way ANOVA revealed statistically significant time ($p < 0.01$), passage number ($p < 0.01$), and time × passage number interaction ($p < 0.05$) effects. When MCF-7 cells were added to passage 15 BPAECs (solid bars), gap area increased and peaked at 12 h after MCF-7 cell addition. After 12 h, gap size became reduced, so that by 48 h after MCF-7 cell addition there was no statistical difference between mean gap size seen at 4 h after MCF-7 cell addition ($p > 0.05$, Student–Neuman–Keuls test following two-way ANOVA). In contrast, MCF-7 cell addition to passage 30 BPAECs caused gap size to increase by 12 h and remain elevated at 48 h with no statistical difference between mean gap area at 12 and 48 h ($p > 0.05$, Student–Neuman–Keuls test following two-way ANOVA). Gaps did not form between BPAECs when conditioned medium was added (results not shown), an observation that has been reported by others in HUVECs with both breast cancer cells [6] and osteosarcoma cells [7]. Finally, analysis of the mean numbers of MCF-7 cells per field was consistent with equal plating of the tumor cells, as there were no statistical differences between the numbers of MCF-7 cells added to the young and old BPAECs in the co-cultured samples (data not shown).

Since in vitro aged BPAECs differ in so many ways from lower passage BPAECs, it was possible that the response to MCF-7 cell addition could be elicited by a non-specific mechanism, such as possible mechanical trauma secondary to the addition of a second cell type, and was not due to the addition of the MCF-7 breast cancer cells per se. To test whether or not the response was specific to MCF-7 cell addition, we performed experiments using CFDASE-labeled 184A1 cells, a non-malignant human mammary epithelial cell line. Labeled 184A1 and MCF-7 cells were added to young and old BPAECs grown on chamber slides. Then 24 h later, samples were fixed. There was no difference in gap area in control BPAEC samples without added cells and samples with added 184A1 cells, while gap area was significantly increased in both the young and old BPAECs after MCF-7 cells were added. For example in one experiment, mean gap areas ± S.D. were 1220 ± 1328 μm^2 in passage 35 BPAECs without added cells and

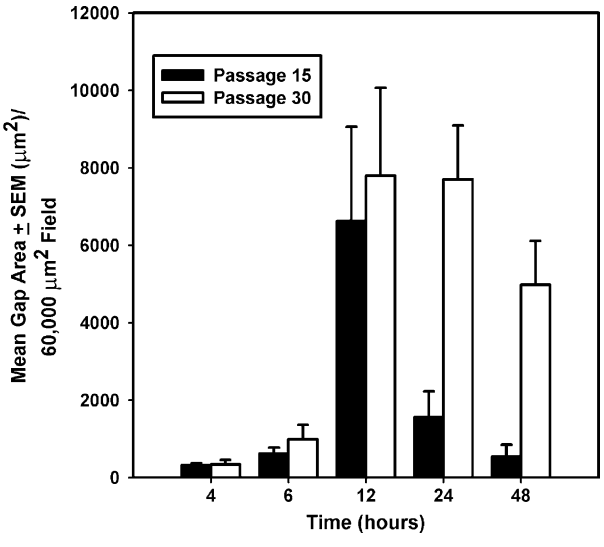
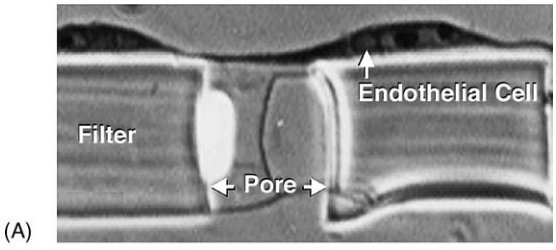


Fig. 3. Time course of gap presence between young and old BPAECs after MCF-7 cell addition. Mean gap area was determined by tracing gaps in digital images of passage 15 BPAECs (solid bars) and passage 30 BPAECs (empty bars) after MCF-7 cell addition. Results of a two-way ANOVA demonstrated statistical significance for passage number ($p < 0.01$), time ($p < 0.01$) and passage number × time interaction ($p < 0.05$) effects.



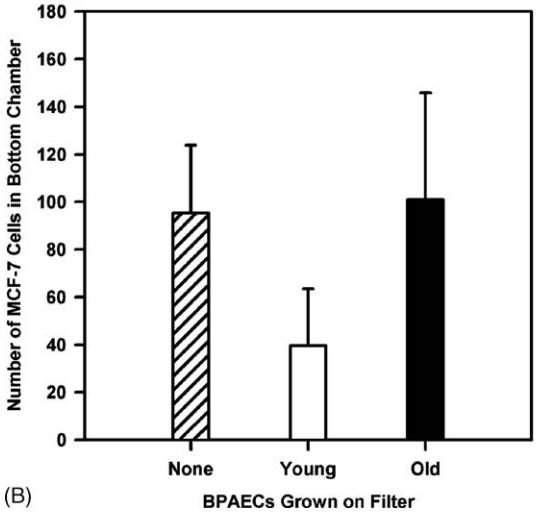


Fig. 4. Transmigration of BPAECs by MCF-7 cells in transwell chambers. (A) BPAECs cover matrigel-coated porous filters in transwell chambers. (B) Numbers of MCF-7 cells crossing filters covered by passage 14 (empty bars) and passage 35 (solid bars) BPAECs and filters without BPAECs (diagonal bars).

$903 \pm 1049\ \mu m^2$ in passage 35 BPAECs with added 184A1 cells ($p > 0.05$). In contrast, the mean gap area was 8524 ± 5790 in passage 35 BPAECs after MCF-7 cell addition ($p < 0.05$ when compared to passage 35 BPAEC controls and samples with added 184A1 cells). These experiments supported the notion that the response of in vitro aged BPAECs to MCF-7 cell addition was specific to adding the cancer cells.

Because it seemed reasonable that larger and persistent gaps between endothelial cells would permit increased MCF-7 cell transmigration, studies were then performed to determine if more MCF-7 cells crossed Matrigel™-coated porous filters covered with old BPAECs as compared to young BPAECs. As shown in Fig. 4A, when endothelial cells were plated onto Matrigel™-coated porous filters in the upper well of transwell chambers, the endothelial cells covered the pores. In the more recent lots of bioinvasion chambers, endothelial cells consistently formed monolayers on both the top and the underside of the filters regardless of the in vitro age of the BPAECs. Twenty-four hours after CFDASE-labeled MCF-7 cells were added to the top wells of the transwell chambers, more MCF-7 cells were found on the underside of the filter and plate bottom when the filters were covered with old BPAECs as opposed to young BPAECs (see Fig. 4B). Furthermore, coating the filters with old BPAECs failed to provide any detectable barrier to MCF-7 cell transmigration, as there was no statistical difference between the numbers of MCF-7 cells crossing filters without BPAECs and those covered with old BPAECs ($p > 0.05$, Student–Neuman–Keuls test following one-way

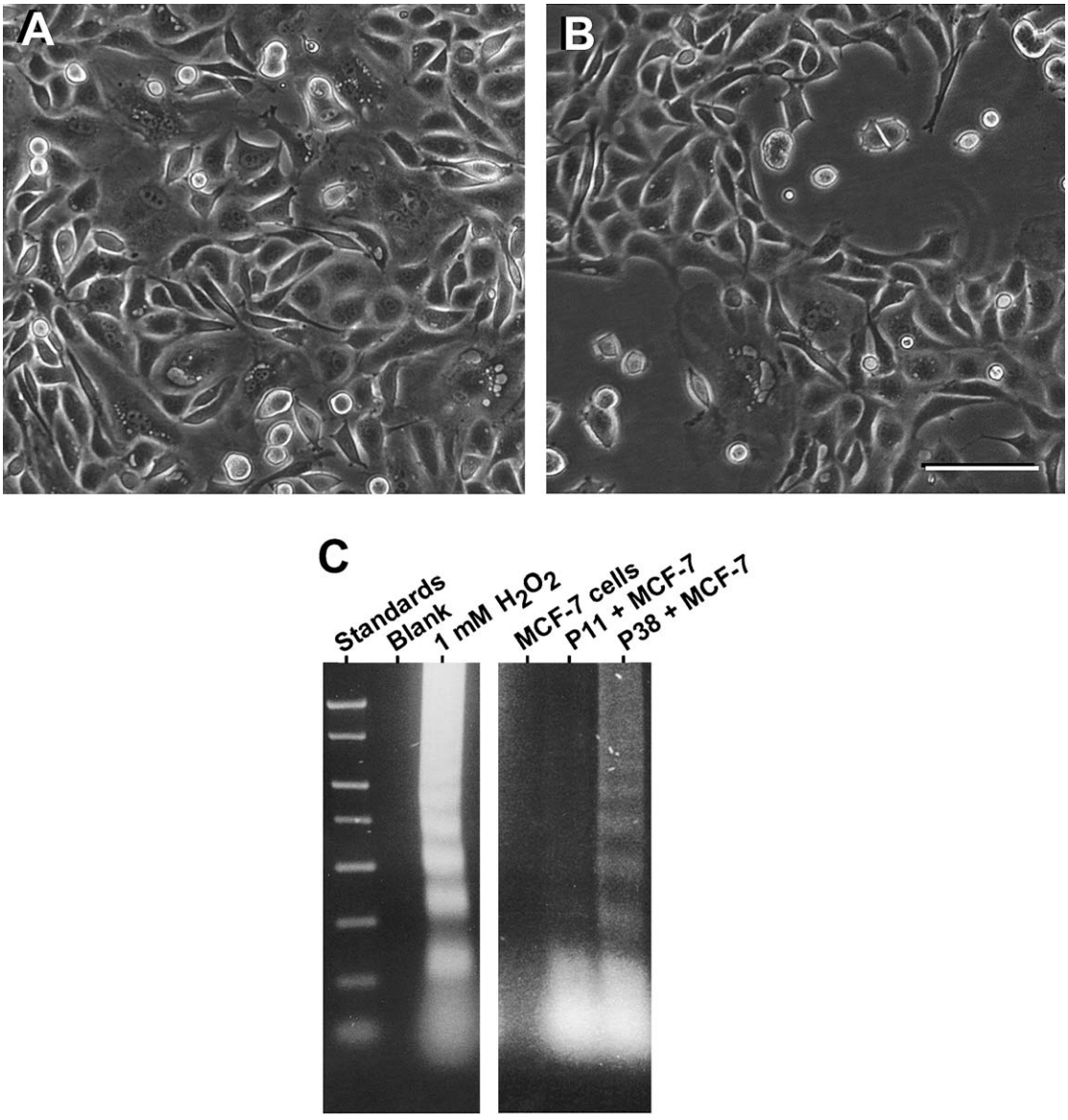


Fig. 5. Effect of MCF-7 cell addition on DNA fragmentation. (A) Few gaps are seen 24 h after MCF-7 cell addition to young BPAECs. (B) Large gaps are seen 24 h after MCF-7 cell addition to old BPAECs (bar equals 100 μm). (C) DNA ladder is present in samples containing peroxide treated BPAECs and old BPAECs 24 h after MCF-7 cell addition. No DNA ladder is seen in young BPAECs 24 h after MCF-7 cell addition.

ANOVA). In contrast, young BPAEC monolayers significantly increased the barrier properties above that of the Matrigel™-coated filters alone ($p < 0.05$, Student–Neuman–Keuls test following one-way ANOVA).

Finally, studies were performed to determine whether or not MCF-7 cell addition to old BPAECs induced a greater degree of endothelial cell apoptosis, in comparison to MCF-7 cell addition to young BPAECs, perhaps accounting in part for the in vitro age-related differences in gap area between neighboring endothelial cells. First, MCF-7 cells were added to young and old BPAECs grown in culture flasks, and digital images were obtained (using the 10× objective of the microscope) prior to harvesting 24 h after MCF-7 cell addition. As shown in Fig. 5A and B, MCF-7 cell addition to young cells (passage 11) caused few gaps in comparison to the large gaps seen in the endothelial monolayer of old cells (passage 38). Agarose gel analysis (Fig. 5C) showed the presence of DNA ladders in lanes containing the old BPAECs to which the MCF-7 cells were added and BPAECs 4 h after the addition of 1 mM hydrogen peroxide (positive control), but not in the lanes containing the young BPAECs to which MCF-7 cells had been added or MCF-7 cells plated alone. The presence of a DNA ladder in old BPAECs after MCF-7 cell addition was consistent with results of the annexin-V binding assay. As seen in Fig. 6, the addition of MCF-7 cells to old BPAECs caused more apoptotic cells per microscopic field as detected by the binding of fluorescently labeled annexin-V to BPAECs (there was a 2.7-fold increase in the number of BPAECs that were labeled by the fluorescent annexin-V). Results from the caspase-3 activation assay were varied. After performing four assays (data not shown), two showed higher levels of caspase-3 activity in the old BPAECs after MCF-7 cell addition, while the remaining two assays showed higher caspase-3 levels in the younger BPAECs after MCF-7 cell addition.

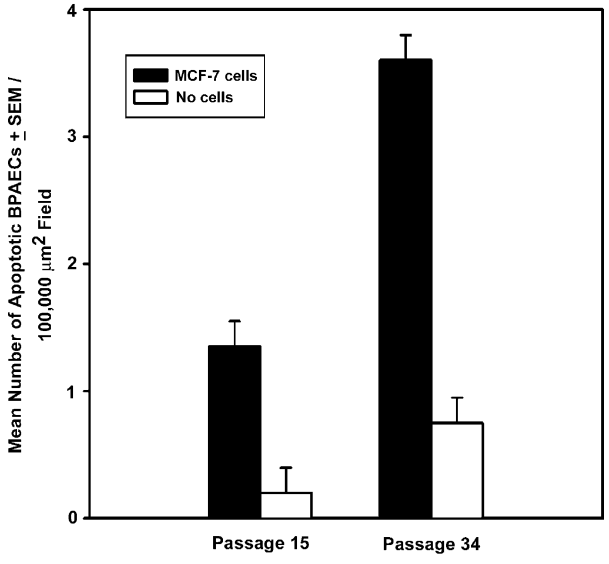


Fig. 6. Effect of MCF-7 cell addition on numbers of apoptotic endothelial cells. Mean number of apoptotic endothelial cells per microscopic field was determined by counting fluorescent annexin-V labeled young (passage 15 shown) and old (passage 34 shown) endothelial cells with (solid bars) and without (empty bars) MCF-7 cell addition. Statistical significance by two-way ANOVA was achieved ($p < 0.01$); Student–Neuman–Keuls method showed no difference between young BPAECs with and without MCF-7 cell addition ($p > 0.05$) and statistical significance between old BPAECs with and without MCF-7 cell addition ($p < 0.05$).

4. Discussion

The novel finding reported here was that the response of BPAECs to MCF-7 cell addition is dependent upon the in vitro age of the endothelial cells. When MCF-7 breast cancer cells were added to young, lower passage BPAECs, transient gaps occur in the endothelial monolayer. In young BPAECs, gaps formed and peaked in size by 12 h after MCF-7 cell addition, then closed. This contrasted to the formation of large gaps that persisted for at least 48 h after addition of MCF-7 cells to old, in vitro aged BPAECs. Our results also showed increased passage of MCF-7 cells across transwell filters covered with old BPAECs compared to filters covered with young BPAECs. Finally, data obtained from DNA fragmentation and annexin-V binding assays, but not caspase-3 activation assays, supported the notion that apoptosis contributed to persistent gaps between old BPAECs after MCF-7 cell addition.

In the literature, two mechanisms by which endothelial cells respond to cancer cell addition have been identified in previous co-culture studies. One mechanism [4,5] involves reorganization of VE- and N-cadherin proteins in HUVECs during cancer cell transmigration and results in N-cadherin-dependent resealing of the monolayer over the transmigrated cancer cell. The second mechanism identified in cancer-endothelial co-culture studies involves cancer-induced endothelial apoptosis. Kebers et al. [6] found that the addition MCF-7 cells to HUVECs caused apoptosis, while apoptosis was not seen when either cancer cell conditioned culture medium or non-malignant mammary epithelial cells were added to HUVECs. Similar results were observed when osteosarcoma cells were added to HUVECS, leading to the conclusion that endothelial apoptosis required direct contact with the cancer cells [7].

In the studies reported here, it appears that the first mechanism in which endothelial cells reseal the monolayer may be consistent with our findings when MCF-7 cells were added to young, lower passage BPAECs. However, studies regarding the role of cadherins in reestablishment of the endothelial monolayers are in progress. The second mechanism in which MCF-7 cells cause endothelial apoptosis appears to prevail when MCF-7 cells were added to the old BPAECs.

Our findings from DNA fragmentation and annexin-V binding assays support increased susceptibility of old BPAECs to apoptosis after MCF-7 cell addition. Indeed, enhanced sensitivity of aged endothelial cells to apoptotic stimuli has been previously reported. Hoffmann et al. [12]

found that HUVECs aged in culture were more susceptible to apoptosis induced by both tumor necrosis factor alpha and oxidized low density lipids, but that the aged endothelial cells per se did not have enhanced intrinsic apoptotic activity under basal conditions.

However, in our experiments performed to assess the effect of MCF-7 cell addition on activation of caspase-3, a key protease activated in several apoptotic pathways, the results were equivocal as no consistent activation of caspase-3 was found after the addition of MCF-7 cells to old BPAECs. Of potential relevance, apoptosis has been reported to occur in some cell types [13–15] in the absence of caspase-3 activation. Perhaps, MCF-7 cell-induced apoptosis in old BPAECs occurs by a similar caspase-3 independent mechanism.

Also, we found that MCF-7 cell conditioned media failed to produce gap formation. This is consistent with the findings of Kebers et al. [6], who reported that MCF-7 cell conditioned medium failed to cause apoptosis in HUVECs. In contrast, MCF-7 cells have been reported to release a soluble form of Fas ligand into conditioned medium that induced apoptosis in osteoblast precursor cells [16]. But perhaps the level of soluble Fas ligand was not high enough in our MCF-7 cell conditioned medium to induce apoptosis in the aged BPAECs, or HUVECs in the case of the studies by Kebers et al. [6] and McEwen et al. [7].

Another explanation for the differences in the in vitro age-related responses to MCF-7 cell addition, including the crossing of porous filters covered by young and old BPAECs, may relate to changes in the extracellular matrix associated with a higher degree of senescent endothelial cells in the old BPAEC population. Though we did not determine the percentage of senescent cells in the older BPAEC populations, the numbers of endothelial cells having the characteristic senescent cell phenotype, i.e. large cells with abnormal nuclei, was increased. Work by others [17] has implicated the extracellular matrix and soluble factors produced by senescent fibroblasts as promoters of tumor growth, but not the growth of normal cells. Perhaps similar factors produced by senescent endothelial cells also alter characteristics of MCF-7 cells and contribute to the in vitro age-related differences in endothelial responses to breast cancer cell addition reported here.

Our findings have potential implications for endothelial aging, which is not always directly associated with chronological age per se, but to factors that contribute to enhanced mitosis of endothelial cells secondary to injury. The role of endothelial cell injury in vascular disorders was proposed many years ago and has been most thoroughly elucidated in atherosclerosis. Injurious factors that include hypertension, smoking [18], high serum lipid levels [19], and increased catecholamines [20] are proposed to injure the endothelium and as a consequence enhance aging by increasing mitotic divisions. Pertinent to the relevance of findings using the in vitro aging endothelial model is that a number of characteristics of the old, higher passage BPAECs have been described in endothelial cells of atherosclerotic plaques [21]. Others [22,23] have reported large endothelial cells, which have been called large multinucleated variant endothelial cells, in the vicinity of the plaques.

More recently, endothelial injury has been proposed to be an important factor in the pathology and progression of other disorders. Indeed research findings support a role for endothelial injury in cancer metastasis (reviewed in [24]). For example, bleomycin-treated mice develop more metastases after the injection of fibrosarcoma cells if the tumor cells are injected soon after bleomycin-induced endothelial injury in comparison to after endothelial repair [25,26].

Although we have found that breast cancer cells cross in vitro aged endothelial cells more readily, we do not know if breast cancer cells will cross aged endothelium more readily and facilitate metastasis in vivo. However, preliminary studies show that MCF-7 breast cancer cells induce gaps in both young and aged human microvascular endothelial cells from the lung. Gap size is significantly larger at 24 and 48 h after breast cancer cell addition to the aged human endothelial cells compared to cells with lower population doubling numbers (data not shown), providing support for our observations on BPAECs. Work is planned to further define the time course of the effects on aged human endothelial cells. Finally, studies are underway to determine if endothelial cells isolated from the lungs of young and old rats exhibit similar responses as those observed in the BPAECs.

Acknowledgments

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Responses of In-vitro Aged Endothelial Cells to Breast Cancer Cell Addition: Role of Endothelial-produced Reactive Oxygen Species in Intercellular Gap Formation and Cell Death

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Abstract

Previous work shows that the addition of breast cancer cells to old endothelial cells in culture causes persistent gaps to form between neighboring endothelial cells. In contrast, breast cancer cell addition to young endothelial cells causes transient gaps. Persistent gap formation between the old endothelial cells is accompanied by increased cancer cell invasion in transwell chambers. The purpose of this study was to investigate two possible causes of these in-vitro age related endothelial differences in responses to breast cancer cell addition. First, young endothelial cells may form transient gaps in response to breast cancer cells, in contrast to persistent gaps, because of more rapid proliferation rates of the younger cells that close gaps more quickly. Secondly, young endothelial cells may be less sensitive to cell death caused by reactive oxygen species (ROS) known to be produced by cancer cells, while ROS-induced old endothelial cell death contributes to persistent gaps. Here we report that bovine pulmonary artery endothelial cells (BPAECs) exhibit in-vitro age related differences to the addition of MCF-7 breast cancer cells that involve the production of and sensitivity to ROS, and not differences in cell proliferation. Old BPAECs are more sensitive to hydrogen peroxide than young cells. Furthermore, in co-culture studies, ROS production is increased by the endothelial cells, and not the MCF-7 breast cancer cells. Gap formation between neighboring BPAECs can be partially blocked by pre-treatment with antioxidants. These findings support the hypothesis that ROS production by both endothelial cells and breast cancer cells contributes in part to gap formation by causing the endothelial cells to die.

Key words: Endothelial cells, MCF-7 cells, cell aging, cell culture

Introduction

Breast cancer is a devastating disease, as well as being the most common form of cancer in women. For the year 2006, it is predicted that 214,640 new cases will be identified in the United States, while 41,430 people will die from the disease (Jemal et al, 2006). Death from breast cancer, as well as many solid tumors, usually results from metastatic disease. Prior to colonizing distant organs, a key event in the metastatic cascade is the extravasation of cancer cells, a process in which endothelial cells are breached as the cancer cells cross the vascular barrier (Weis et al, 2005). Although some progress is being made in identifying mechanisms involved in extravasation, e.g. VEGF-induced src activation in endothelial cells and related changes in vascular permeability (Weis et al, 2004), the process as whole awaits elucidation. Increased knowledge regarding factors that facilitate transmigration of breast cancer cells across the endothelial barrier is needed to better understand extravasation and prevent death from breast cancer.

Recently, we have found that the in-vitro age of endothelial cells alters the responses of endothelial cells to breast cancer cells in ways that may be relevant to cancer extravasation (Merkle et al, 2005). In cell culture studies in which MCF-7 breast cancer cells are added to bovine pulmonary artery endothelial cells (BPAECs) of passages 10 to 16, referred to as "young" endothelial cells, transient gaps form between neighboring endothelial cells. These gaps appear by 4 hours after cancer cell addition, peak in size at 12 hours, then completely close by 48 hours after cancer cell addition. This is in contrast to large gaps that remain by 48 hours after the MCF-7 breast cancer cells are added to BPAECs of passages 30 to 36, or "old" endothelial cells. Furthermore, when BPAECs are grown on porous filters in transwell chambers, there is a four-fold increase in the numbers of MCF-7 cells transmigrating old BPAECs, in comparison to young BPAECs.

Due to the important role of transendothelial cell migration in cancer extravasation and metastasis, these age-related observations warrant additional study. In this report, we have investigated two potential mechanisms to explain the differences in the responses of young and old BPAECs to MCF-7 cell addition. The first mechanism involves age-related differences in mitotic indexes. It has been well documented that lower passage cultured cells have higher proliferation rates (Macieira-Coelho et al, 1966a, 1966b). Perhaps, the gap closure is due to mitotic division after gap formation induced by the addition of the cancer cells. Young BPAECs may fill in the gaps due to a higher mitotic rate, compared to old BPAECs. Secondly, as our earlier study (Merkle et al, 2005) implicates apoptosis as a mechanism for the differences in breast cancer-induced gaps between young and old BPAECs, perhaps there are differences in the sensitivity of the endothelial cells to reactive oxygen species (ROS) generated by the cancer cells. Breast cancer cells and other cancer cell types produce ROS including hydrogen peroxide (Nathan and Szatrowski, 1991). The production of ROS by cancer cells has been proposed to be a mechanism of cancer-induced injury to the endothelium (Paduch et al, 2005).

Indeed in this report we find that BPAECs exhibit in-vitro age related differences to the addition of MCF-7 breast cancer cells that involved the production of and sensitivity to ROS, rather than differences in mitotic indices. An unanticipated finding is that in the co-culture studies, ROS production by endothelial cells is increased while its production by the MCF-7 breast cancer cells is not. Increased production of ROS by endothelial cells as a response to the addition of cancer cells is to our knowledge a previously unreported observation. Our findings may be relevant to extravasation and cancer metastasis.

Materials and Methods

Cell Cultures

BPAECs from a private stock (Schaeffer et al, 1992) and MCF-7 cells (American Type Culture Collection, Manassas, VA) were used. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (Hyclone Laboratories, HyClone Laboratories, Logan, UT), penicillin (100 units/mL), streptomycin (0.1 mg/mL), and gentamicin (50 g/mL). BPAECs were aged by passing confluent cells in Hank's EDTA solution with trypsin and removing half of the cell population. BPAEC passages 10 through 16 are defined as "young" BPAECs, whereas passages 30 through 36 were defined as "old" BPAECs. For the experiments, BPAECs were plated on either gelatinized and fibronectin-coated 8-well plastic chamber slides or gelatinized 24- or 96-well plates.

Reagents and Assay Kits

The 5-bromo-2'-deoxyuridine (BrdU) kit was obtained from Roche Diagnostics (Indianapolis, IN). Rhodamine-conjugated phalloidin, the Amplex Red kit, the carboxyfluorescein diacetate succinimdyl ester (CFDASE) kit (Vybrant^{TM} CFDA SE Cell Tracer Kit), and 5-(and 6-) chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) were purchased from Molecular Probes (Eugene, OR). Catalase, -tocopherol (vitamin E), N-acetyl cysteine (NAC) and mitomicin C were purchased from Sigma Chemical Company (St. Louis, MO).

Mitosis Studies

To determine BPAEC proliferation rates, young and old BPAECs were plated on chamber slides (2,000 cells per well), then fixed 24, 48 and 72 hours later. Numbers of BPAECs per microscope field were determined for each time point.

Two types of experiments were performed to determine if the proliferation rate of young BPAECs accounted for gap closure by 48 hours after the addition of MCF-7 breast cancer cells.

First, young and old BPAECs were grown to confluence on gelatinized and fibronectin-coated 8-well chamber slides. After pre-incubating the BPAEC monolayers in 10 M BrdU in DMEM, CFDASE-labeled MCF-7 cells were added (2500 cells per well) to monolayers. Controls included BPAECs with no MCF-7 cell addition, co-cultures without BrdU, and sparsely plated BPAECs incubated with BrdU as a positive control for BrdU staining. Samples were fixed in formaldehyde 24 and 48 hours after MCF-7 cell addition, then stained with the anti-BrdU antibody provided in the kit and a goat anti-mouse secondary antibody. Samples were viewed using phase contrast optics combined with fluorescence illumination and the 40X objective of a Leica DMIRB microscope. Digital images were captured using a SPOT™ camera (Diagnostic Instruments, Inc., Sterling Heights, MI). The number of BrdU-positive BPAECs were determined in each image and mean numbers of BrdU-positive BPAECs per group calculated.

Secondly, young and old BPAEC monolayers in chamber slides were pre-incubated in mitomicin C (10 μg/mL DMEM), or an equivalent volume of saline in DMEM as controls, for 30 minutes then returned to plain culture medium. CFDASE-labeled MCF-7 cells were added 24 hours later. Samples were fixed 48 hours after MCF-7 cell addition and stained with rhodamine-phalloidin. Companion black-and-white and color fluorescent images were obtained, and mean gap areas determined in the black-and-white images by measuring "black" gap areas using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).

ROS Studies

These studies began by measuring ROS production in co-cultures of BPAECs and MCF-7 breast cancer cells. MCF-7 breast cancer cells were added to monolayers of young and old BPAECs in 96-well plates (20,000 breast cancer cells per well), and were co-cultured for either 1 or 24 hours. In the initial experiments, ROS were assayed by loading both BPAECs and MCF-7

cells with CM-H_2DCFDA (10 uM) after 24 hours of co-culturing. Later, BPAEC ROS production was measured by loading the BPAECs with CM-H_2DCFDA prior to addition of MCF-7 cells, then measuring ROS an hour after MCF-7 cell addition. Controls were BPAECs without MCF-7 cell addition. For determination of ROS production by the MCF-7 cells alone, MCF-7 cells were loaded with CM-H_2DCFDA then ROS measured one hour after adding the loaded MCF-7 cells to BPAECs. Controls were dye-loaded MCF-7 cells alone. ROS-associated fluorescence was measured using a Tecan SpectraFluor plate reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. ROS concentration was expressed as relative fluorescence units (RFU).

To specifically measure hydrogen peroxide, we added Amplex Red reagent (50 μM) to monolayers of young and old BPAECs grown in 96-well plates, then the medium was changed to Krebs-Ringer phosphate containing glucose (KRPG) at the same time MCF-7 breast cancer cells were added (20,000 cells per well). Four hours later, the KRPG was collected from each well and transferred to wells of clear bottom, black-walled plates for hydrogen peroxide measurement. Fluorescence was measured on the plate reader at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The concentration of hydrogen peroxide was determined by comparison to a standard curve of known hydrogen peroxide concentrations.

To determine the effects of hydrogen peroxide on young and old BPAECs, monolayers of endothelial cells grown in gelatinized 24-well plates were treated with hydrogen peroxide at varied concentrations (1 μM, 10 μM, 100 μM, 250 μM, and 500 μM) for 48 hours. Cell numbers were determined and results expressed as percentages of control samples without added hydrogen peroxide. To determine if antioxidants protected endothelial cells from hydrogen peroxide-induced injury, catalase (final concentration 100 U/ml), NAC (15 mM) and vitamin E

(final concentration 50 uM) were added 30 minutes prior to the addition of hydrogen peroxide to young and old BPAECs. After 48 hours the number of attached, living cells were counted microscopically.

Finally, to examine the effects of antioxidants on breast cancer-induced gaps between endothelial cells, young and old BPAECs were grown as monolayers on 8-well chamber slides, then pre-incubated with either catalase, NAC, or vitamin E for 30 minutes prior to adding CFDASE-labeled MCF-7 breast cancer cells. Antioxidants were maintained for 24 hours after addition of the breast cancer cells, then samples were fixed and stained with rhodamine-phalloidin. Samples were viewed and images obtained as described above to measure gap areas from black-and-white images.

Statistical Analysis

Analyses were performed using SigmaStat software (Jandel). One and two-way ANOVAs were performed as appropriate, and followed by the Student-Newman-Keuls post hoc test, as indicated. Statistical significance was defined as $p < 0.05$.

Results

Mitosis Studies

To determine whether or not differences in the rates of mitosis in young and old BPAECs accounted for gap closure after MCF-7 breast cancer cells were added to the BPAECs, we first sought to verify that the younger cells did indeed have a higher proliferation rate as has been reported for other cell types (Macieira-Coelho et al, 1966a, 1966b). As shown in Figure 1A, the number of young BPAECs doubled, in contrast to the old BPAECs in which the number of cells at 48 hours after plating did not differ from the number plated.

Next, studies using BrdU-incorporation were performed to determine if newly divided endothelial cells closed the gaps. Dual phase and fluorescent images were taken at 24 and 48 hours after MCF-7 breast cancer cell addition to BPAEC monolayers, and data were collected by counting the total number of fluorescent nuclei, as an indication of recently divided endothelial cells. The data were analyzed by two-way ANOVA. As shown in Figure 1B, there was no statistical difference in mean number of recently divided endothelial cells based on endothelial cell age or on time after breast cancer cell addition. Note that the mean number of BrdU-labeled cells per microscopic field for both the young (black bar) and the old (white bar) BPAECs was less than one endothelial cell. The small number of cells undergoing mitosis in the images suggests that neither the young nor the old BPAECs were dividing during the time period of study to fill in the gaps in the monolayer triggered by MCF-7 cell addition. Furthermore, there was no difference between numbers of recently divided endothelial cells in samples in which MCF-7 breast cancer cells had been added or not added (data not shown).

To further test the hypothesis, young and old BPAECs were treated with mitomicin C to prevent cell division before the addition of MCF-7 breast cancer cells. The results of these studies, as shown in Figure 1C, did not support the hypothesis that endothelial cell mitosis was filling the MCF-7 cell-induced gaps in the endothelial monolayers. In the samples containing young and the old BPAECs treated with mitomicin C, gap size was actually smaller than gap size in the untreated cells. The mechanism for this observation was unknown, though it appeared that mitomicin C may have been associated with enhanced cell spreading or endothelial cell enlargement. Regardless, both studies failed to support differences in mitotic index as the mechanism for gap closure in young BPAECs and gap persistence between old BPAECs after MCF-7 cell addition.

ROS Studies

Studies were performed to test the hypothesis that ROS were involved in the differential responses of young and old BPAECs to MCF-7 breast cancer cell addition. First, intracellular ROS levels and hydrogen peroxide production were measured in samples of young and old BPAECs after the addition of MCF-7 cells. To determine if the addition of MCF-7 breast cancer cells to BPAEC monolayers increases ROS production, two sets of experiments were performed. In the first, MCF-7 breast cancer cells pre-labeled with CM-H_2DCFDA were added to young and old BPAECs grown as monolayers. One hour later, ROS produced by the MCF-7 cells were measured. As shown in Figure 2A, the addition of MCF-7 cells to BPAEC monolayers actually decreased the ROS produced by the cancer cells ($p<0.05$). This result was not anticipated. Furthermore, MCF-7 cell addition to the young BPAECs caused higher ROS levels than addition of MCF-7 cells to old BPAECs ($p<0.05$).

In the second set of experiments, young and old BPAECs were grown as monolayers and pre-labeled with the CM-H_2DCFDA dye to allow measurement of ROS production by endothelial cells. Unlabeled MCF-7 breast cancer cells were then added to the BPAECs. As seen in Figure 2B, the addition of MCF-7 cells to BPAECs caused significant increases in ROS concentration in both the young and the old BPAECs ($p<0.05$, Student-Newman-Keuls post hoc test following the ANOVA). Furthermore, production of ROS in young BPAECs was also significantly higher than in the old BPAECs ($p<0.05$) both before and after MCF-7 breast cancer cell addition ($p < 0.05$). In comparison to media of old BPAECS, increased levels of hydrogen peroxide were also detected in the media of young BPAECs, both before and after MCF-7 cell addition ($p<0.05$, Student-Newman-Keuls test following the ANOVA).

As with ROS production, there was a statistically significant increase in production of hydrogen peroxide in both the young and the old BPAECs after MCF-7 cell addition ($p < 0.05$; Figure 3A). Young and old BPAECs were also treated with varying concentrations of hydrogen peroxide to determine if older BPAECs were more sensitive to it. As seen in Figure 3B, young BPAECs (black bars) appeared unaffected by doses as high as 500 μM hydrogen peroxide as cell viability remained unchanged. Old BPAECs (white bars) were sensitive to hydrogen peroxide concentrations as viability was reduced in a dose dependent manner ($p<0.05$, Student-Newman-Keuls following the two-way ANOVA).

The final experiments were conducted to determine if antioxidants could block breast cancer-induced gap formation between endothelial cells and hydrogen peroxide-induced BPAEC death. As shown in Figure 4A, pretreatment of BPAECs with catalase partially reduced gap size after MCF-7 cell addition to both young and old BPAECs, whereas pre-treatment with NAC and vitamin E, Figures 4B and 4C respectively, reduced gap size in young BPAECs only. Furthermore, treatment with catalase and NAC improved survival of both young and old BPAECs after the addition of 500 M hydrogen peroxide (Figures 5A and 5B). Although treatment of BPAECs with vitamin E did not improve survival of either young or old BPAECs exposed to 500 M hydrogen peroxide, over all our findings suggested a role for ROS in the varied responses of young and old BPAECs to MCF-7 breast cancer cell addition.

Discussion

We sought to test hypotheses regarding differences related to mitosis and ROS to explain in vitro age-related responses of old BPAECs to MCF-7 breast cancer cell addition in comparison to MCF-7 cell addition to young BPAECs. Although there were differences in the division rate of young and old BPAECs during log-phase growth in sub-confluent cultures, the

higher mitotic index of young BPAECs does not appear to account for endothelial cell gap closure. There were very few newly divided BPAECs at 48 hours after MCF-7 cell addition to confluent cultures of either the young or old BPAECs, and the mean numbers of newly divided BPAECs in these samples were not statistically different. Additionally, the mitomicin C experiments did not provide support for cell proliferation as a mechanism of gap closure. Instead, an ROS-associated mechanism may account for the in-vitro age-related differences in the responses of BPAECs to MCF-7 cell addition. Increased sensitivity of aged BPAECs to hydrogen peroxide-induced endothelial cell death was identified. Further, the addition of MCF-7 breast cancer cells increased both hydrogen peroxide in the culture medium and ROS production in both co-cultures containing young and old BPAECs. An unanticipated finding was that ROS production was increased in the BPAECs, and not the breast cancer cells. Finally, addition of antioxidants reduced the MCF-7 cell-induced gaps between BPAECs, demonstrating the involvement of ROS in gap formation.

These findings warrant additional comment because gaps between endothelial cells may have relevance to cancer metastasis. Previous work supports the idea that breachment of the endothelial barrier occurs as cancer cells enter and exit the vasculature. Steps delineating the process have been described from intravital microscopy studies of rodents (Chambers et al, 1995), as well as in-vitro studies with co-cultured cancer cells and endothelial cells (Sandig et al, 1997; Voura et al, 1998). The in-vitro work by Sandig et al (1997) described sequential changes in VE- and N-cadherin mediated intercellular contacts as melanoma cells transmigrate endothelial cells, establishing the importance of these proteins. More recent experimental evidence supports involvement of the cadherins as a response to vascular endothelial growth factor (VEGF) secreted by cancer cells. VEGF has been shown to modulate the transendothelial

migration of MDA-MB-231 breast cancer cells by regulating brain microvascular endothelial cell permeability (Lee et al, 2003). Furthermore, by injecting VEGF-secreting cancer cells into mice, investigators show VEGF-activation of src and subsequent VE-cadherin changes that enable the cancer cells to cross the endothelial barrier by disrupting the VE-cadherin- -catenin complex in the lung and other organs (Weis et al, 2004).

Since apoptosis is known to be induced by ROS and tumor cells are known to produce large amounts of ROS such as hydrogen peroxide (Nathan and Szatrowski, 1991), studies were performed to determine if ROS could account for the age related differences in breast cancer cell-induced gap formation between endothelial cells. Hydrogen peroxide was measured in culture supernatants using the hydrogen peroxide-specific Amplex Red reagent, which is oxidized to the fluorescent product resorfun by hydrogen peroxide, but not other ROS (Song et al, 2001). Though hydrogen peroxide is produced intracellularly by the reaction of superoxide anion with superoxide dismutase, it can be measured extracellularly because it is freely membrane permeant (Ushio-Fukai and Alexander, 2004). Furthermore, superoxide released at the cell surface is reported to be converted to hydrogen peroxide (Song et al, 2001).

Intracellular ROS levels were measured using a membrane-permeant probe (CM-H_2DCFDA) which is converted to an impermeant form by intracellular esterases. This probe is converted to a fluorescent product by a variety of ROS, including nitric oxide, peroxynitrite, superoxide, potassium oxide, lipid peroxides, in addition to hydrogen peroxide (Hempel et al, 1999). Hence, compared to the Amplex Red reagent, it is a broad-spectrum indicator of intracellular levels of reactive nitrogen and oxygen intermediates.

Using these assays, we found that the addition of MCF-7 breast cancer cells did indeed increase both ROS in the supernatant and intracellularly. This was anticipated because cancer

cells are known to produce ROS at levels equal to those produced by stimulated polymorphonuclear neutrophils (Nathan and Szatrowski, 1991), and hydrogen peroxide production by MCF-7 cells has been documented (Policastro et al, 2004). The surprise was that the predominant increase in ROS was within the endothelial cells, and not the cancer cells, as predicted. MCF-7 breast cancer cell-induced increases in ROS in the BPAECs can be explained. It is recognized that a wide variety of peptide growth factors and cytokines induce ROS production in many cell types, including endothelial cells, in which ROS serve as intermediates in signal transduction (Finkel, 1998). Though we did not determine the specific mechanisms involved here, it is plausible that the cytokines VEGF and transforming growth factor beta (TGF) are responsible, since these peptides are secreted by MCF-7 cells and endothelial cells express the corresponding receptors. MCF-7 cells, other breast cancer cell lines and normal breast epithelial cells secrete biologically active levels of VEGF in-vitro (Weigand et al, 2005). This cytokine has been demonstrated to induce ROS in cultured endothelial cells via activation of VEGF receptor 2 (VEGFR-2 or KDR) and to initiate angiogenic signaling (Colavitti et al, 2002). Similarly, MCF-7 cells secrete significant amounts of TGF in vitro (Malet et al, 2001), while endothelial cells express TGF receptors RI, RII and RIII (Hirai and Kaji, 1992; Morello et al, 1995). Endothelial cell stimulation with TGF induces a marked increase in ROS production via NADPH oxidase and results in an altered cytoskeletal organization (Hu et al, 2005). Endothelial ROS has also been demonstrated to mediate loss of VE-cadherin function, resulting in inter-endothelial gap formation (van Buul et al, 2005). Taken together, these observations suggest that VEGF and TGF produced by MCF-7 cells may be responsible for the increased endothelial ROS production reported here.

Our finding that the older endothelial cells have increased sensitivity to ROS is consistent with the work of others. In human endothelial cells, increased oxidative stress has been associated with premature aging and senescence-associated cell death (Unterluggauer et al, 2003). Furthermore, studies in primary cultures of human chondrocytes demonstrated that these cells grown from aged humans (≥ 50 years) were significantly more sensitive to oxidant-induced cell death than those from younger humans (18-49 years). This was concluded to result from dysregulation of the glutathione (GSH) antioxidant system, with old cells demonstrating a 4-fold higher level of GSSG (oxidized GSH) than young cells (Del Carlo and Loeser, 2003). Many animal studies have reported an aging-associated decline in tissue GSH levels along with increased GSSG levels, indicating that the antioxidant system in aged animals is less effective in countering oxidative stress. (reviewed in Maher, 2005). These changes may be due to declining GSH synthesis, since key enzymes in its production, i.e. glutamate cysteine ligase and glutathione synthase, are decreased in some tissues of aged rats (Liu et al., 2004). In addition, catalase, an enzyme that converts hydrogen peroxide to H_2O and O_2 has been reported to be reduced in liver tissue of aging rats (Chen et al., 2006) and in aging human skin (Shin et al., 2005).

Taken together, it is likely that our observation of an increased susceptibility of aged endothelial cells to hydrogen peroxide-induced cell death is due to an aging-associated decrease in antioxidant activity. Additionally, studies to test the roles of VEGF and TGF produced by the MCF-7 breast cancer cells in endothelial ROS production are indicated, as are continued studies of the role of VEGF in cancer transmigration of endothelial cells.

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Figure Legends

Figure 1. Mitosis Studies. (A) Differences in mitosis rates between sub-confluent young and old BPAECs. (B) BrdU positive endothelial cells in confluent samples of young (black bars) and old (white bars) BPAECs after MCF-7 breast cancer cell addition. (C) Endothelial gap area 48 hours after MCF-7 breast cancer cell addition in confluent samples of young (black bars) and old (white bars) BPAECs with and without mitomicin C pre-treatment.

Figure 2. Reactive Oxygen Species. (A) Differences in ROS in MCF-7 breast cancer cells alone (white bar) or 1 hour after addition to young (black bar) and old (gray bar) BPAECs. (B) Differences in ROS in young and old BPAECs with (white bars) and without addition (black bars) of MCF-7 breast cancer cells. ROS was measured after 1 hour of incubation.

Figure 3. Hydrogen Peroxide Studies. (A) Hydrogen peroxide levels in samples of young and old BPAECS with (black bars) and without (white bars) the addition of MCF-7 breast cancer cells. Hydrogen peroxide was measured after 4 hours of incubation. (B) Sensitivity of young (black bars) and old (white bars) BPAECs after exposure to varied concentrations of hydrogen peroxide for 48 hours.

Figure 4. Effect of Antioxidants on MCF-7 Cell-Induced Gap Area in Young and Old BPAECs. (A) BPAECs treated (black bars) and untreated (white bars) with catalase. (B) BPAECs treated (black bars) and untreated (white bars) with N acetyl cysteine. (C) BPAECs treated (black bars) and untreated (white bars) with vitamin E. Cells were treated for 48 hours then gap area determined.

Figure 5. Effect of Antioxidants on Viability of Young and Old BPAECs 48 hours after Hydrogen Peroxide Treatment. (A) Effects of catalase. (B) Effects of n-acetyl cysteine. (C) Effects of Vitamin E.

Figure 1

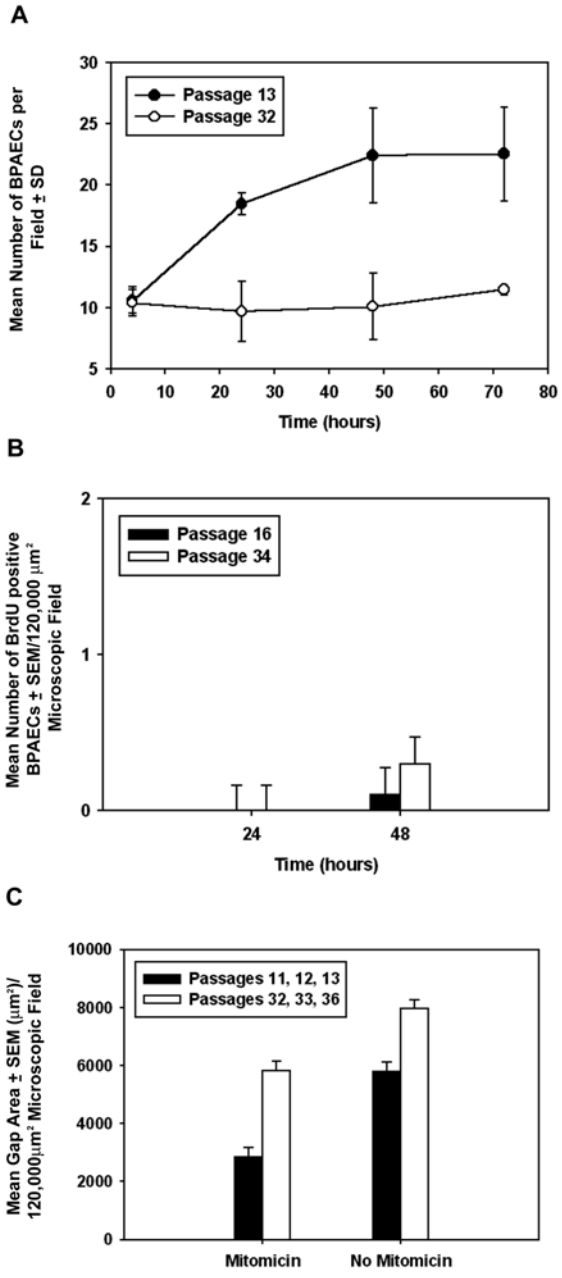

A
Mean Number of BPAECs per Field ± SD
Passage 13
Passage 32
Time (hours)
B
Mean Number of BrdU positive BPAECs ± SEM/120,000 µm² Microscopic Field
Passage 16
Passage 34
Time (hours)
C
Mean Gap Area ± SEM (µm²)/ 120,000µm² Microscopic Field
Passages 11, 12, 13
Passages 32, 33, 36
Mitomicin
No Mitomicin

Figure 2

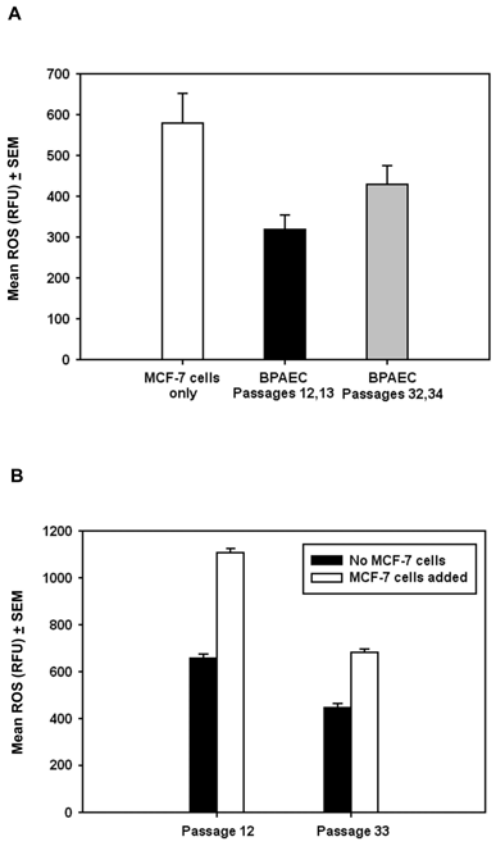

A
Mean ROS (RFU) ± SEM
700
600
500
400
300
200
100
0
MCF-7 cells only
BPAEC Passages 12,13
BPAEC Passages 32,34
B
Mean ROS (RFU) ± SEM
1200
1000
800
600
400
200
0
No MCF-7 cells
MCF-7 cells added
Passage 12
Passage 33

Figure 3

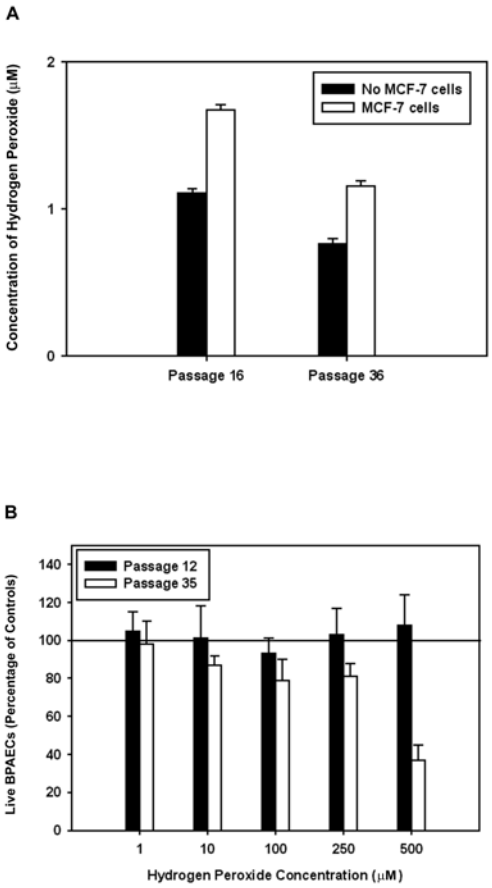

A
Concentration of Hydrogen Peroxide (μM)
2
1
0
No MCF-7 cells
MCF-7 cells
Passage 16
Passage 36
B
Live BPAECs (Percentage of Controls)
140
120
100
80
60
40
20
0
Passage 12
Passage 35
1
10
100
250
500
Hydrogen Peroxide Concentration (μM)

Figure 4

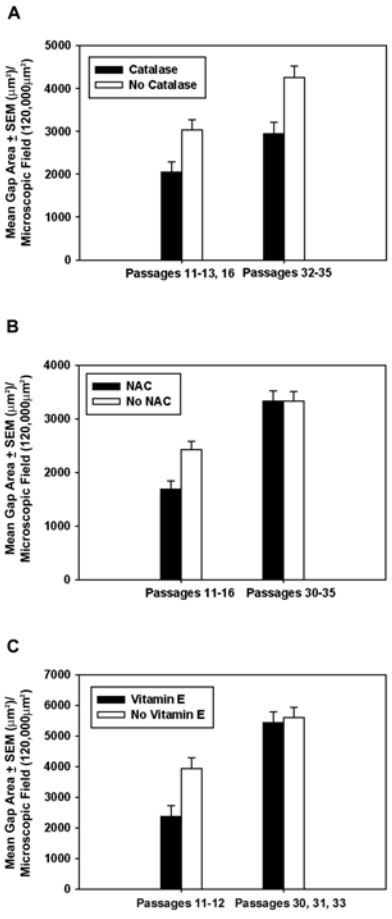
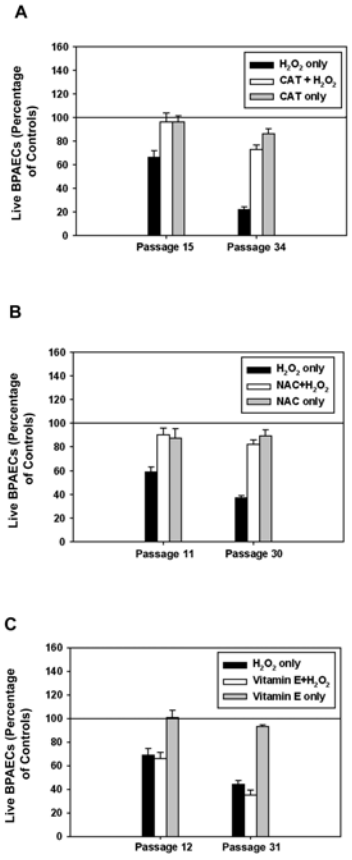

A
Catalase
No Catalase
Mean Gap Area ± SEM (μm²)/ Microscopic Field (120,000μm²)
5000
4000
3000
2000
1000
0
Passages 11-13, 16
Passages 32-35
B
NAC
No NAC
Mean Gap Area ± SEM (μm²)/ Microscopic Field (120,000μm²)
4000
3000
2000
1000
0
Passages 11-16
Passages 30-35
C
Vitamin E
No Vitamin E
Mean Gap Area ± SEM (μm²)/ Microscopic Field (120,000μm²)
7000
6000
5000
4000
3000
2000
1000
0
Passages 11-12
Passages 30, 31, 33

Figure 5


A
Live BPAECs (Percentage of Controls)
160
140
120
100
80
60
40
20
0
H_2O_2 only
CAT + H_2O_2
CAT only
Passage 15
Passage 34
B
Live BPAECs (Percentage of Controls)
H_2O_2 only
NAC+H_2O_2
NAC only
Passage 11
Passage 30
C
Live BPAECs (Percentage of Controls)
H_2O_2 only
Vitamin E+H_2O_2
Vitamin E only
Passage 12
Passage 31